FATTY ACID COMPOSITION OF MUSCLE PHOSPHOLIPIDS FROM CALVES, AND GROWING AND MATURE STEERS FED PROTECTED SAFFLOWER OIL

INTRODUCTION

THE RELATION between fats and heart disease is controversial; nevertheless many consumers believe a strong correlation exists between high cholesterol (the saturated fat content of ruminant products) and coronary heart disease, and that polyunsaturated fats should be substituted for saturated fats in the diet whenever possible. However, the polyunsaturated fatty acid content in older ruminants seldom exceeds 4% of the fat fraction because the polyunsaturated fatty acids present in plant materials in the diet are hydrogenated in the rumen by microorganisms to form saturated and monounsaturated fatty acids (Reiser, 1951; Shorland et al., 1957).

Recently Australian investigators (Scott et al., 1970; 1971) developed a process of feeding ruminants oil supplements that were protected from hydrogenation in the rumen; animals showed marked increases in the linoleic acid content of the depot fats and phospholipids (Scott et al., 1971; Faichney et al., 1972; Cook et al., 1972). With this process, the Animal Physiology and Genetics Institute, and the Nutrition Institute, ARS, USDA, Beltsville, Maryland, produced calves, and growing and mature steers with increased linoleic acid in the depot fats. Meat samples from these animals were made available to us for chemical analysis. The history of the calves (Wrenn et al., 1973) and steers (Dinius et al., 1974) has been reported.

Stability properties of these meat samples are our primary interest. The triglycerides from the adipose tissues are the primary cause of deterioration during freezer storage (Sulzbacher and Gaddis, 1968), whereas the muscle phospholipids are the major contributors to the oxidative deterioration of cooked (Younathan and Watts, 1960) and freeze-dried meats (El-Gharbawi and Dugan, 1965; Chipault and Hawkins, 1971).

The stability of rendered subcutaneous fats from calves (Ellis et al., 1974) and

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from growing and mature steers (Kimoto et al., 1974), as well as the palatability data on freezer-stored roasts from these animals will be reported elsewhere.

We report herein the 2-thiobarbituric acid (TBA) values, tocopherol content, and fatty acid composition of the phospholipids from the rump roasts of these animals.

EXPERIMENTAL

ALL MEAT SAMPLES were obtained from the Animal Physiology and Genetics Institute, and the Nutrition Institute.

Calves

Two groups of 4-day-old bull calves, four in each group, were fed normal milk (NM) or polyunsaturated milk (PUM) from cows fed safflower oil-casein-formaldehyde (Plowman et al., 1972), both supplemented with 486 mg α-tocopheryl acetate per calf per day, for 10 wk. The groups were then further divided into smaller groups of two animals and fed either safflower oil-casein-formaldehyde (protected, P) or safflower oil-casein (unprotected, U) as supplements to the basal diet for 8 wk (Wrenn et al., 1972; 1973). The dietary groups thus formed were: NM-U, NM-P, PUM-U and PUM-P.

Growing and mature steers

Two sets of six growing steers each were fed for 6.5 wk on a regular basal diet supplemented with 10 or 20% concentrations of either safflower oil-casein-formaldehyde (P), or safflower oil-casein (U). Three animals were assigned to each treatment. The 8-month-old growing steers were fed a restrictive diet (2% of body weight per day) and ate all the feed offered. The average weight at slaughter was approximately 230 kg and 240 kg for the steers on the 10% and 20% treatments, respectively. Two mature steers were fed for 7 wk on the basal diet supplemented with 11% safflower oil-casein-formaldehyde, a third animal was fed safflower oil-casein, and a fourth mature steer was fed 6% sodium caseinate (C). The average weight of the 18-month-old mature steers was 500 kg.

Every animal received 20 mg of d-α-tocopheryl acetate per day (Dinius et al., 1974).

Fatty acid analysis

A portion from the round (rump roast) from each animal was excised 1 day after slaughter and stored at $-18°C$ until needed. About 1 lb of the frozen rump roast was cut. Since the rump roast consists primarily of semimembranosus and semitendinosus muscles, with lesser amounts of biceps femoris and adductor, and minor amounts of gracilis muscles (Tucker et al., 1952), a representative sample of these muscles is obtained only by cutting against the

grain of the roast. The partially thawed sample was cubed and, after removal of all visible fat tissues, was ground twice through a 1/8-in. plate, and hand mixed thoroughly. Samples of 30g and 10g were taken, in duplicate, for phospholipid and tocopherol determinations, respectively. The lipids were extracted in a blender with 300 ml and then 200 ml MeOH-CHCl$_3$ (1:2) according to the procedure of Folch et al. (1957). The combined extract was placed in a separatory funnel and mixed with 0.2 of its volume with water. The mixture was refrigerated for 1–2 hr, then allowed to stand at room temperature for 1 hr; two clear layers separated with an interfacial fluff. The lower layer was placed in a 1-liter round-bottom flask with 0.2 ml of 0.1% butylated hydroxytoluene (BHT) in CHCl$_3$ (v/v). The flask was placed in a 30–40°C water bath and the solvents were removed with a rotary evaporator under house vacuum. Lipids were separated on a silicic acid column with MeOH-CHCl$_3$ (1:20), MeOH-CHCl$_3$ (1:1), and MeOH by the procedure of Hornstein et al. (1961). The first fraction contains the neutral lipids (primarily triglycerides) and the last two fractions contain the phospholipids.

For calf samples, the phospholipid fractions, containing about 20 mg lipids, were placed in 50 ml test tubes and solvents removed with a jet of N$_2$. The residues were saponified with EtOH-KOH at 65°C and the fatty acids converted to their methyl esters with 5% HClO$_4$ in MeOH at 65°C (Schmitt and Wynn, undated).

For the growing and mature steer samples, the MeOH-CHCl$_3$ (1:1) and MeOH fractions from the separation of the lipids on silicic acid were combined and the fatty acids of the phospholipids were converted into their methyl esters by a modification of the transesterification procedure of Luddy et al. (1968), consisting of the addition of 2–3 drops of anhydrous benzene to the reaction mixture (Luddy, 1972). Conversion of the MeOH-CHCl$_3$ (1:20) or neutral lipid fraction into methyl esters was by the procedure of Luddy et al. (1968). The carbon disulfide extract of the methyl esters was transferred to a 5-ml vial and the solvent removed with a jet of N$_2$. Methyl heptadecanoate, 0.2 ml of a 1% solution in CHCl$_3$ (w/v), was added to the residue as an internal standard. The methyl esters were analyzed by gas-liquid chromatography as previously described (Ellis et al., 1974); in addition the programmed runs were held at the upper limit (210°C) for 10–13 min.

All of the methyl esters had retention times identical to those of standard compounds except 20:3, for which no standard was available. A semi-log plot of the carbon number against the ratio of the elution temperature of standard methyl esters to the elution temperature of the internal standard, 17:0, indicated that the compound was 20:3 (Schmitt and Wynn, undated).

This compound has been reported as a constituent of phospholipids from beef muscle (Hornstein et al., 1961; O'Keefe et al., 1967) and assignment was made on this basis. The multiplying factor, which was the ratio of peak height of the standard (17:0) to peak height of the standard methyl esters, was used to normalize the peak heights in the extracts, and the factor calculated for 20:4 was also used for 20:3. Numerous minor peaks before the 20:4 peak and three peaks appearing after the 20:4 peak represented 2% and 6% or less, respectively, of the total peak heights for the phospholipids. These peaks represented unidentified compounds and were not included in the present analysis.

Fatty acid compositions from MeOH-CHCl$_3$ (1:1), and MeOH fractions were combined for the calf phospholipid results.

Tocopherols

Tocopherols were isolated from the muscle tissues by a modification of the procedure of Erickson and Dunkley (1964). A mixture of 10g of ground lean tissues and 15 ml of absolute EtOH was placed in a 50 ml centrifuge tube and mixed thoroughly. Stirring was continued after the addition of 1 ml of 1N HCl and the mixture placed in a water bath at 60°C for 5 min with occasional stirring. While still warm the mixture was transferred into a 250 ml flat-bottom flask with 15 ml of hexane, shaken mechanically for 20 min, returned to the 50 ml centrifuge tube and centrifuged for 5–10 min. The solvents were decanted, and the extraction procedure was repeated on the residue two more times, with the mixture being shaken for 10 instead of 20 min. The extracts were placed in a separatory funnel, diluted with 45 ml of distilled or deionized water and 5 ml of 1N HCl and extracted with three 35 ml portions of hexane. The hexane extracts were combined and the volume reduced to 30 ml with a jet of N$_2$. After drying over Na$_2$SO$_4$ the hexane extracts were reduced to 20 ml with an N$_2$ jet; 10

ml aliquot was used for the tocopherol determination.

TBA numbers on ground meat stored at 3°C

About 1 lb of rump roast frozen at −18°C for 6 months for the growing steers and 1.5 months for the mature steers, was ground twice through a 1/8-in. plate and hand mixed thoroughly. Twelve 30-g aliquots of meat were taken from each sample and rolled into balls. Three balls were placed in each of four aluminum foil flat-bottom boats. These were placed in pyrex trays, covered with aluminum foil and stored at 3°C. After 0, 1, 2 and 3 days the three balls of ground meat in each boat were combined, thoroughly hand mixed and the 2-thiobarbituric acid (TBA) number was determined essentially by the method of Tarladgis et al. (1960). A simple still was used instead of the Kjeldahl apparatus, and the Kjeldahl flask was heated with a Meker burner. Absorbance of the TBA pigment was recorded at 538 nm on a Cary model 14 spectrophotometer. Absorbance was multiplied by K to give the TBA number in mg of malonaldehyde per 1,000g of sample. Our K value was 8.1, somewhat higher than the 7.8 reported by Tarladgis et al. (1960).

Data for the fatty acid composition and the TBA numbers from the growing steers were treated by analysis of variance (Snedecor, 1956).

RESULTS & DISCUSSIONS

Calves

Table 1 shows average fatty acid composition of the phospholipids from the rump roasts of four pairs of experimental and one pair of commercial calves. Calves fed polyunsaturated milk or protected safflower oil showed a trend toward greater incorporation of 18:2 into the phospholipid of meat than control calves

fed normal milk and unprotected safflower oil. The total polyunsaturated fatty acid content and 18:2 levels of the phospholipids were dependent on the diet in the order PUM-P = NM-P > PUM-U > NM-U. Commercial calf samples of unknown history were obtained fresh from the killing floor of a local slaughter plant. These samples were included for purposes of comparison.

During analysis of the fatty acid methyl esters by gas-liquid chromatography two extraneous compounds were observed which represented about 10% of the total peak heights. One peak eluted before methyl palmitate (16:0) and the other between methyl heptadecanoate (17:0) and methyl stearate (18:0). Extraction of these compounds with hexane after saponification of the phospholipids with alcoholic KOH, indicated that they were not fatty acids. They formed 2,4-dinitrophenylhydrazone derivatives with a maximum of 345 nm in CCl$_4$, which was in the region for the absorption maxima of alkanals (Gaddis et al., 1959), indicating they were saturated or unconjugated unsaturated carbonyl compounds. The retention time for the peak eluting between 17:0 and 18:0 was identical to that of stearic aldehyde and the peak eluting before 16:0 was believed to be palmitic aldehyde. The source of the aldehydes may be plasmalogens. The transesterification procedure of Luddy et al. (1968) and Luddy (1972) does not liberate aldehydes from phospholipids. With Luddy's procedure, the results of the analysis of the fatty acid composition of the phospholipids from the left rump

Table 1—Average tocopherol content and fatty acid composition (wt %) of phospholipids from rump roasts of calves[a]

	PUM-P[b]	PUM-U	NM-P	NM-U	Commercial (#1, #2)
16:0	14.9	14.6	16.8	15.7 ± 1.8	15.4
16:1	0.7	1.3 ± 0.3	0.7	1.6 ± 0.7	0.6 ± 0.1
18:0	16.8 ± 1.2	16.5	16.3	15.9 ± 2.1	16.5
18:1	7.2 ± 0.4	11.0	7.0	14.2 ± 1.1	22.5 ± 1.6
18:2	38.8	34.5	38.3	29.0	26.6 ± 1.8
18:3	0.4 ± 0.1	1.0	0.4	1.5 ± 0.4	1.4 ± 0.1
20:3	2.3	3.0	2.6 ± 0.5	3.9	2.9 ± 0.3
20:4	18.9 ± 1.6	18.1	17.9 ± 1.1	18.2	14.1
% Phospholipids, wet wt	0.71 ± 0.02	0.67 ± 0.01	0.71 ± 0.05	0.78 ± 0.01	0.86 ± 0.02
Tocopherols, µg/g	6.6 ± 1.0	5.2 ± 0.3	4.4 ± 0.3	4.7 ± 0.1	2.5 ± 0.1
% Saturated fatty acids	31.7	31.1	33.1	31.6	31.9
% Monounsaturated fatty acids	7.9	12.3	7.7	15.8	23.1
% Polyunsaturated fatty acids	60.4	56.6	59.2	52.6	45.0

[a] Standard deviation is shown if more than ±5% of the mean from two animals per treatment.
[b] Diet of normal milk (NM) or polyunsaturated milk (PUM), followed by diet supplemented with protected (P) or unprotected (U) safflower oil

Table 4—TBA numbers for ground rump roasts stored at 3°C[a]

Days after storage	Growing steers[b]				Mature steers	
	10% P[c]	20% P	10% U	20% U	P	U, C
0	1.3 ± 0.3	1.0 ± 0.1	1.2 ± 0.3	1.5 ± 0.4	1.0 ± 0.1	1.3 ± 0.1
1	4.1	3.7 ± 0.1	3.1 ± 0.3	3.8 ± 0.6	4.1 ± 0.3	4.1 ± 0.3
2	6.3 ± 0.2	5.9 ± 0.2	5.1 ± 0.3	6.0 ± 0.8	6.1 ± 0.1	6.4 ± 0.3
3	7.5 ± 0.2	7.6 ± 0.3	6.6 ± 0.8	7.8 ± 1.0	7.6 ± 0.1	8.1 ± 0.5

[a] TBA number = mg malonaldehyde per 1,000g of sample. Mean values and standard deviation of the mean

[b] Analysis of variance showed differences were not significant (P < 0.05) among the four treatments or between protected and unprotected diet supplements.

[c] Diet supplemented with protected (P) or unprotected (U) safflower oil or sodium caseinate (C)

incorporation of 18:2 into the phospholipids than into the triglycerides of growing and mature steers.

TBA determination on ground meat stored at 3°C

Growing steers fed unprotected safflower oil had greater variations and somewhat lower TBA numbers than steers fed protected safflower oil, but these differences were not significant (P < 0.05). Mature steers fed unprotected safflower oil or sodium caseinate also had greater variations but slightly higher average TBA numbers than the steers fed protected safflower oil (Table 3).

TBA determinations by Keskinel et al. (1964) on fresh ground lean tissues of beef stored at 5°C indicated that increase in TBA numbers was greatest after storage for 2 days. On this basis, oxidation of ground lean tissues from growing and mature steers should have progressed far enough after 3 days at 3°C to indicate degrees of difference without significant bacterial growth. Oxidation of lipids in ground meat depends primarily on the degree of unsaturation and on amounts of lipid, antioxidants and heme catalysts (Tappel, 1962). The last should be comparable for the meat samples used, with some differences in tocohperol levels, and marked differences in degree of unsaturation of the lipids.

During the early stages of oxidation the TBA value correlates linearly with peroxide value, diene conjugation and oxygen uptake (Kenaston et al., 1955; Tarladgis and Watts, 1960; Dahle et al., 1962); its use for quantitative determination of lipid oxidation may be meaningful only during this period (Kwon and Olcott, 1966). Up to peroxide levels of 2,000 meg/kg, methylene-interrupted polyunsaturated fatty acids at the same peroxide values had TBA values that were approximately in the ratio 1:2:3:4, for the triene, tetraene, pentaene and hexaene, respectively, with none for linoleic acid (Dahle et al., 1962). These data show that TBA value depends on the profile of polyunsaturated (triene or greater unsatu-

ration) fatty acids that are oxidized and may not correlate with the degree of oxidation of the total lipids. For this reason the visibly more rapid change in meat color from pink to brown for the ground meat samples from the animals fed protected over those fed unprotected safflower oil may indicate differences in degree of lipid oxidation, but not TBA numbers.

The incorporation of 18:2 into the phospholipids appears to be more rapid than into the triglycerides of muscle tissues. However the phospholipids represent less than 1% of the weight of the wet muscle tissue and will not have much impact on increasing the polyunsaturated fatty acid content of the fat fraction of meat products. For the oxidative deterioration of freeze-dried and cooked meat products for which the phospholipids have been implicated, an increase in the 18:2 level of the phospholipids without a corresponding increase in the level of protection by antioxidants will result in decrease stability of the meat product.

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roast of one sample from each of the four dietary groups was similar to the results from the saponification-esterification of the right rump roasts. The liberation of aldehydes, therefore, did not affect the analysis of the fatty acid composition of the phospholipids.

For the experimental animals tocopherol content varied from 4.2–7.3 µg/g which was much higher than the 2.4–2.6 µg/g level for the commercial calves. This difference was attributed to the supplemental vitamin E fed the calves the first 10 wk.

Growing and mature steers

Table 2 summarizes the average fatty acid composition of the phospholipids from growing and mature steers. Statistical analysis of the data shows significant differences (P < 0.01) in concentrations of 16:1, 18:1, 18:2, 18:3 and 20:3 in the phospholipids from animals receiving protected or unprotected safflower oil in the diet; the protected treatment produced higher levels only for 18:2. With either 10% or 20% P in the diet the 18:2 level was about 40% of the fatty acid contents, nearly twice as much as in the phospholipids of animals fed the unprotected diet. The increase in 18:2 was counterbalanced by decreases in the other unsaturated fatty acids except 20:4, which was not significantly different. Similar results were reported by Cook et al. (1972). Differences were similar for mature steers fed protected safflower oil in comparison to those fed unprotected material or sodium caseinate.

Incorporation of tocopherols in the muscle tissues of growing and mature steers varied from 2.8–4.9 and from 2.7–3.1 µg/g, respectively, and were lower than the values for the experimental calves. Although the deposition of dietary tocopherol in animal tissue is rather inefficient (Machlin, 1962), tissue levels can be increased by increasing tocopherols in the diet over a period of time. Differences in the tocopherol level in the diet were reflected in the muscle tissues of ewes (Buchanan-Smith et al., 1969) and calves (Eaton et al., 1958; Decker and Hill, 1957).

Table 3 summarizes the 18:2 composition of the neutral lipids (primarily triglycerides) from the rump roasts of growing and mature steers. Comparison between Tables 2 and 3 shows significant differences for the 18:2 content between the P treatments for the triglycerides (Table 3) but not for the phospholipids (Table 2). There was also a significant difference in 18:2 composition between the U treatments for the phospholipids but not for the triglycerides, although the triglyceride mean value for the 20% U was slightly higher than that for the 10% U treatment. The 18:2 composition of the phospholipids (38.8%) for the mature steers on the P treatment was similar to that for the growing steers (40.3%), although the corresponding composition of 18:2 for triglycerides was markedly different. These results suggest a more rapid

Table 2—Average tocopherol content and fatty acid composition (wt %) of phospholipids from rump roasts of growing and mature steers

	Growing steers[b]				Mature steers[c]	
	10% P[a]	20% P	10% U	20% U	P	U, C
16:0	15.9	15.0	16.4	15.5	15.8	16.2 ± 1.1
16:1	0.9b	0.8b	2.4c	1.3b	0.7	1.3 ± 0.3
18:0	17.5b,c	17.0b,c	16.5b	17.7c	14.1	14.1 ± 1.5
18:1	7.3b	6.4b	23.5c	18.4c	8.7	21.0
18:2	40.3b	40.2b	19.8c	26.1d	38.8	22.1
18:3	0.6b	0.5b	2.3c	1.6d	0.5	1.3 ± 0.3
20:3	3.0b,d	2.3b	3.9c,d	4.0c	2.9 ± 0.5	4.2
20:4	14.5	16.5	15.2	15.4	18.5 ± 1.1	19.8
% Phospholipids, wet wt	0.64 ± 0.03	0.65 ± 0.03	0.66 ± 0.02	0.65 ± 0.02	0.64 ± 0.04	0.60 ± 0.03
Tocopherols, µg/g	3.0 ± 0.3	3.3 ± 0.2	3.8 ± 1.1	3.5 ± 0.1	3.0 ± 0.2	2.7 ± 0.1
% Saturated fatty acids	33.4	32.0	32.9	33.2	29.9	30.3
% Monounsaturated fatty acids	8.2	7.2	25.9	19.7	9.4	22.3
% Polyunsaturated fatty acids	58.4	60.8	41.2	47.1	60.7	47.4

[a] Diet supplemented with protected (P) or unprotected (U) safflower oil, or sodium caseinate (C)
[b] Horizontal values on the same line not bearing the same letter differ significantly (P < 0.05).
[c] Standard deviation is shown if more than ±5% of the mean from two animals per treatment.

Table 3—18:2 composition (wt %) of triglycerides from rump roasts of growing and mature steers[a]

	Growing steers[b]				Mature steers	
	10% P[c]	20% P	10% U	20% U	P	U, C
18:2	8.6d ± 1.7	13.8e ± 1.6	2.9f ± 0.4	4.0f ± 1.3	4.4 ± 0.2	2.6 ± 0.4
% Lipids, wet wt	1.3 ± 0.4	1.3 ± 0.5	1.5 ± 0.6	1.4 ± 0.5	6.9 ± 0.3	5.9 ± 1.8

[a] Mean values and standard deviation of the mean. Fatty acid composition based on: 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3
[b] Horizontal values on the same line not bearing the same letter for the growing steers differ significantly (P < 0.01).
[c] Diet supplemented with protected (P) or unprotected (U) safflower oil, or sodium caseinate (C)